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Oxidative Stress and Aromatic Hydrocarbon Response of Human Bronchial Epithelial Cells Exposed to Petro- or Biodiesel Exhaust Treated with a Diesel Particulate Filter

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ABSTRACT

The composition of diesel exhaust has changed over the past decade due to the increased use of alternative fuels, like biodiesel, and to new regulations on diesel engine emissions. Given the changing nature of diesel fuels and diesel exhaust emissions, a need exists to understand the human health implications of switching to "cleaner" diesel engines run with particulate filters and engines run on alternative fuels like biodiesel. We exposed well-differentiated normal human bronchial epithelial cells to fresh, complete exhaust from a diesel engine run (1) with and without a diesel particulate filter and (2) using either traditional petro- or alternative biodiesel. Despite the lowered emissions in filter-treated exhaust (a 91–96% reduction in mass), significant increases in transcripts associated with oxidative stress and polycyclic aromatic hydrocarbon response were observed in all exposure groups and were not significantly different between exposure groups. Our results suggest that biodiesel and filter-treated diesel exhaust elicits as great, or greater a cellular response as unfiltered, traditional petrodiesel exhaust in a representative model of the bronchial epithelium.

Key words: in vitro; bronchial; diesel particulate filter; diesel exhaust; CYP1A1; HO-1

Ambient air pollution has recently been identified as the eighth leading cause of death worldwide (Lim *et al.*, 2012) and previous studies have reported that diesel emissions are a leading source of particulate matter air pollution (Chow *et al.*, 1992; Lim *et al.*, 2012; Health Effects Institute, 2013). Human exposure to diesel exhaust (DE) is widespread due to the combustion of diesel fuel for construction, agriculture, mining, and transportation. Inhalation of DE has been associated with chronic lung inflammation and fibrosis (Henderson *et al.*, 1988; Mauderly *et al.*, 1988), allergic immune responses in the lungs (Sydbom *et al.*, 2001), asthma (Pandya *et al.*, 2002; Northridge *et al.*, 1999), lung cancer (IARC, 2012; NIOSH, 1988), and cardiovascular health effects (Lucking *et al.*, 2008; McDonald *et al.*, 2011). Diesel particulate matter (DPM) was also recently classified as a human carcinogen (IARC, 2012). Although the health effects from exposure to traditional forms of diesel combustion exhaust have been well studied, the potential effects from exposure to (1) filtered and (2) biodiesel exhaust have not been extensively evaluated (Swanson *et al.*, 2007). Further, very few studies have evaluated the human airway cell response to filtered biodiesel exhaust, and of the studies that have, most have evaluated the effects of DE

particles alone and not the complete exhaust mixture (Gerlofs-Nijland *et al.*, 2013; Swanson *et al.*, 2009; Schwarze *et al.*, 2013).

The U.S. EPA recently reduced the annual standard for fine particulate matter ($PM_{2.5}$) from 15 to 12 $\mu g/m^3$ in an effort to reduce mortality rates, incidents of heart attacks, stroke, and childhood asthma resulting from exposure (EPA, 2012). In an effort to reduce diesel emissions in particular, the U.S. EPA initiated a "National Clean Diesel Campaign" in 2006, which required refiners to produce low-sulfur diesel fuel (EPA, 2013). Further, the campaign requires engine producers to reduce particle emissions by 90% in on-road engines by 2007, and in off-road engines by 2010 (EPA, 2013). After-treatment technologies like diesel particulate filters are often used to meet these emission reduction requirements. However to date, the number of studies that have assessed the toxicity of "clean" DE (i.e., emissions that are treated with a diesel particulate filter) is few, and existing studies present conflicting results (Karthikeyan *et al.*, 2013; McDonald *et al.*, 2004; Rudell *et al.*, 1999).

Concern over rising petroleum costs and limited resources have resulted in a push toward more stable, renewable energy supplies. With the widespread availability of crop surpluses in the U.S., the use of biodiesel is forecasted to increase as a fuel in the transportation, mining, industrial, and agricultural sectors (Chaudhuri, 2013). Between 2010 and 2011, biodiesel production in the United States increased almost 3-fold from an estimated 350 million gallons to 1 billion gallons. Biodiesel production capacity for 2014 is forecasted to be 2.2 billion gallons, with 112 biodiesel plants currently in operation (Chaudhuri, 2013; EIA, 2013). Although biodiesel emissions have been well studied and characterized, the human health impacts from exposure to these emissions are uncertain (Swanson *et al.*, 2007).

The objective of this work was to assess acute cellular responses following exposure to DE from an engine run with (1) petro- or biodiesel and (2) a diesel particulate filter. We investigated the response of well-differentiated human bronchial epithelial (NHBE) cells following exposure to various forms of DE: with and without a diesel particulate filter and using either traditional petrodiesel or biodiesel fuel. Transcripts associated with oxidative stress (HO-1) and polycyclic aromatic hydrocarbon (PAH) response (CYP1A1) were measured 2 h after exposure. Cytotoxicity was measured as well. To our knowledge, this is the first study to examine oxidative stress and PAH responses in airway cells exposed to complete exhaust from an engine run with (1) petro- or biodiesel and (2) a diesel particulate filter.

MATERIALS AND METHODS

Electrostatic aerosol in vitro exposure system. We used an electrostatic aerosol *in vitro* exposure system (EAVES) that has been described previously (Hawley *et al.*, 2014). A schematic of the EAVES system is shown in Supplementary figure 1. Fresh DE was diluted, charge neutralized (Kr-85 neutralizer, TSI Inc.), conditioned to physiological temperature and humidity (37°C, 80–90% relative humidity), and then drawn through the EAVES chamber at 1.5 l/min. In the cell chamber, charged particles were forced onto the cultures by an electric field that cycled from −4 to +4 kV at constant periodicity (10 s at each polarity). By cycling the polarity of the electric field over time, a net neutral charge of particles was delivered to cell cultures. Aerosol deposition in the EAVES has been characterized previously (Hawley *et al.*, 2014). For particles between 10 and 700 nm in size, the percentage of incoming aerosol deposited per cm^2 of cellular growth area ranged from 0.29% to 0.43% by mass.

Cell culture. Normal human bronchial epithelial (NHBE) cells were obtained by brush biopsy from two healthy, non-smoking human volunteers (EPA, Research Triangle Park, NC) in accordance with a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Cell populations were expanded through two passages in Petri dishes with Bronchial Epithelial Growth Media (BEGM kit; Lonza, Walkersville, MD) before being plated onto collagen-coated, porous, polycarbonate membranes (0.4-μm Snapwell membrane; Corning, Inc., Corning, NY) at a seeding density of approximately 140,000 cells per cm^2. Air–liquid interface (ALI) cultures were carried for a minimum of 21 days (prior to exposure) to allow progressive differentiation into basal, ciliated, and mucin-producing cell types within a pseudo-stratified columnar epithelium (Ross *et al.*, 2007). Mucus production was visually apparent by day 10 of ALI and excess mucus was removed with a gentle saline rinse, every 3 days thereafter.

Cell exposures to DE. Tests were performed using a 4.5-l John Deere 4045H PowerTech Plus diesel engine. The PowerTech Plus engine is a 4-cylinder, common rail, direct injection diesel engine that includes exhaust gas recirculation and an exhaust filter that contains diesel oxidation catalyst and a diesel particulate filter. The diesel oxidation catalyst reacts with exhaust gases to reduce carbon monoxide, hydrocarbons and a portion of the particulate matter (PM). The diesel particulate filter (Tenneco, Grass Lake, MI, model number: TENN NO H0084737A) traps and oxidizes PM via a passive regeneration process that utilizes heat from the exhaust gases. Experiments were performed with and without the combined oxidation catalyst/particulate filter installed. An eddy current dynamometer (Midwest Inductor Dynamometer, 1014A) and dynamometer controller (Dyn-LocIV) were used to maintain a constant torque on the engine at a given speed. All tests were performed at 75% load (107 BHP) and an engine speed of 2400 rpm. Prior to the test campaign, the engine lubrication oil was changed and the same lubrication oil charge was used for all tests.

Well-differentiated NHBE cells (cultured at ALI for a minimum of 21 days) were placed in the EAVES chamber and exposed to fresh, complete DE for a duration of 5, 20, or 60 min ($n = 12$ per treatment group). An experimental matrix is provided in Supplementary table 1. On each test day, the JD 4045H PowerTech Plus diesel engine was brought to steady temperature, load, and speed while running on either ultra-low-sulfur petrodiesel (Team Petroleum, LLC, Fort Collins, CO) or biodiesel (commercial-grade B99 (Supplementary table 2), Suncor Refining, Commerce City, CO) fuel. All tests reported herein were performed at 75% load (107 BHP) at rated speed (2400 rpm). Tests with a diesel particulate filter (*DPF+*; Tenneco, Grass Lake, MI, model number: TENN NO H0084737A) and tests without a diesel particulate filter (*DPF−*) were conducted in a similar manner. For all cell exposures, fresh exhaust was pulled through a dilution tunnel (Bennett *et al.*, 2008) at a ratio of 1:20 of fresh exhaust to supplemental, heated, filtered air. Engine exhaust was pulled from the dilution tunnel through a cyclone with a $PM_{2.5}$ cutpoint (GK2.05, BGI Inc., Waltham, MA) and then into the EAVES cell exposure system. Particle size distributions were measured with a Sequential Mobility Particle Sizer (SMPS+C; Grimm Technologies, Douglasville, GA) immediately upstream of the EAVES for the duration of the cell exposures. Control cells were exposed to the same conditions stated above, except that incoming room air was HEPA filtered to remove >99% of particles. Each exposure condition was repeated on 2 separate days. We compared the response of complete DE-exposed cells with the response

of control cells, 2 h after exposure, as previous work suggests that mRNA production reaches a peak at this time point (Hawley *et al*., 2014).

Characterization of engine exhaust. Emissions were collected directly from the dilution tunnel used to supply fresh exhaust for cell exposures. Mass emissions during each test condition were measured by collecting fresh DE onto pre-weighed Teflon filters, following the methods of Subramanian *et al*. (2004). Elemental/organic carbon content was analyzed by collecting fresh DE onto pre-baked quartz filters. In one line, emissions were collected onto a Teflon filter with a quartz filter in line behind the Teflon filter. In the second line, emissions were collected directly onto a quartz filter alone. The quartz filters were then stored at −80°C until analysis (EC/OC Lab Instrument, Sunset Laboratories Inc.). Elemental, organic, and total carbon emissions were calculated according to NIOSH method 5040. Gaseous emissions measurements were made immediately downstream of the engine exhaust using a heated sample transfer line and a five-gas analyzer (Rosemount Analytical, Houston, TX), which reported levels of total hydrocarbons (THC), oxygen (O_2), carbon monoxide (CO), carbon dioxide (CO_2), and nitrogen oxides (NO_x). Measured gaseous emissions were then multiplied by 0.05 to account for the 20-fold dilution of the exhaust used for cell exposures.

Transcript production in ALI NHBE cells. Combustion-derived particles can sequester cytokines produced by epithelial cells, and a negative bias can occur when these cytokines are quantified using ELISA (Kocbach *et al*., 2008; Seagrave *et al*., 2004; Seagrave, 2008; Totlandsdal *et al*., 2010). Previous work by Wyatt *et al*. suggests that mRNA transcripts can be used as a proxy for cytokine production in NHBE cells (Wyatt *et al*., 2007). Therefore, we chose to quantify levels of mRNA transcripts as indicators for subsequent cytokine production.

We quantified transcripts coding for proteins that characterize cellular oxidative stress (Heme oxygenase-1, HO-1) and PAH-adduct formation (Cytochrome p450 1A1, CYP1A1). All transcript analyses were quantified by RT-PCR (CFX96, Bio-Rad Laboratories, Hercules, CA) according to MIQE guidelines (Bustin *et al*., 2009). Expression profiles for each transcript were normalized to GAPDH (Barber *et al*., 2005). All transcript expression profiles were normalized to controls' expression levels of each transcript.

Cytotoxicity in ALI NHBE cells. Lactate dehydrogenase (LDH) is expressed universally in NHBE cells. The loss of membrane integrity during cell injury and death causes the extracellular release of LDH, which can be used as an indicator of cytotoxicity (Allan and Rushton, 1994). Extracellular LDH was assayed at 2 h post-exposure using a standard kit (Promega Cytotox96 Non-radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI). Percent cytotoxicity was calculated by following the manufacturer's protocol (Promega, 2012).

Statistical analyses. Transcript data were log-transformed to satisfy model assumptions of normality and homoscedasticity. The effects of exposure duration, exposure type, donor phenotype, and experimental repeat (and their interactions) were evaluated relative to the expression of HO-1 and CYP1A1 transcripts and extracellular LDH using one-way ANOVAs. Cell donor and experimental replicate were treated as random effects. Statistical analyses were conducted with SAS software (v9.0, SAS Institute Inc., Cary, NC) with a type I error rate of 0.05.

RESULTS

Exhaust Characteristics

The size distribution by particle count of the DE is shown in Figure 1. This size distribution was relatively stable within a given treatment and between replicates. The count median diameters (CMD) of each exhaust treatment can be seen in Table 1. Particle emissions were different between each exhaust treatment. Combustion of biodiesel produced consistently smaller size distributions (CMDs that were 50% and 58% smaller for DPF− and DPF+ treated, respectively), and fewer in number than petrodiesel PM emissions. A similar trend was observed with the DPF-treated exhaust. DPF-treated (DPF+) exhaust resulted in smaller CMDs (33% and 22% smaller for petro- and biodiesel exhaust, respectively) and particles that were fewer in number than unfiltered exhaust.

Particle mass concentrations of each exhaust treatment are shown in Table 1. Petrodiesel and unfiltered (DPF−) exhaust contained higher $PM_{2.5}$ mass concentrations compared with biodiesel and DPF-treated exhaust, respectively. Use of a DPF reduced $PM_{2.5}$ mass emissions by 96% and 91% for petro- and biodiesel exhaust, respectively. Concentrations of particulate elemental and organic carbon are shown in Table 2 as a function of each exhaust treatment. Elemental carbon emissions were substantially higher in the unfiltered petrodiesel exhaust than in the unfiltered biodiesel exhaust. Concentrations of elemental carbon were greatly reduced in the DPF+ treated exhaust (99% and 97% reductions for petro- and biodiesel exhaust, respectively). Biodiesel exhaust contained 66% (DPF+) and 89% (DPF−) less elemental carbon when compared with unfiltered petrodiesel. Organic carbon content did not differ between fuel types for the filtered exhaust treatment groups. However, organic carbon content was reduced substantially in the unfiltered biodiesel exhaust, as compared with the unfiltered petrodiesel exhaust (50% reduction, by organic carbon mass). The DPF significantly reduced organic carbon content (reductions of 95% and 91% in petro- and biodiesel exhaust, respectively). Gaseous emissions in each exhaust treatment are shown in Table 3. Total hydrocarbons were reduced by 86–87% in the filtered exhaust (petro- and biodiesel exhaust, respectively). Biodiesel exhaust contained fewer total hydrocarbons than petrodiesel exhaust. Although nitrous oxide (NO) was decreased in the filtered exhaust from petrodiesel (−44%) ($p = 0.0014$), NO increased in filtered biodiesel exhaust (10%) ($p = 0.86$). Nitrogen dioxide (NO_2) increased significantly in exhausts from both fuel types, when treated with a DPF (+357% ($p = 0.0011$) and +572% ($p = 0.0225$) for petro- and biodiesel exhaust, respectively). Total NO_x increased slightly in filtered petrodiesel exhaust (1%). On the other hand, DPF treatment caused a 70% increase in NO_x in biodiesel exhaust, mostly attributable to a significant increase in NO_2 (9.55 ppm in DPF+ vs. 1.41 ppm in DPF− exhaust).

Cell Exposures

Levels of DPM mass delivered to NHBE cell cultures during the 5, 20, and 60-min exposures are shown in Table 4. These deposition rates were calculated based on mass concentrations, exposure duration, and the known deposition efficiency in the EAVES (0.29–0.43% of incoming particle mass delivered per cm^2 cellular growth area) (Hawley *et al*., 2014). The PM mass delivered to each exhaust treatment group varied significantly. Within each exhaust treatment exposure group, PM mass delivered increased by 4- (20-min exposure) and 12-fold (60-min exposure). In the highest exposure groups (60-min exposure duration), PM mass

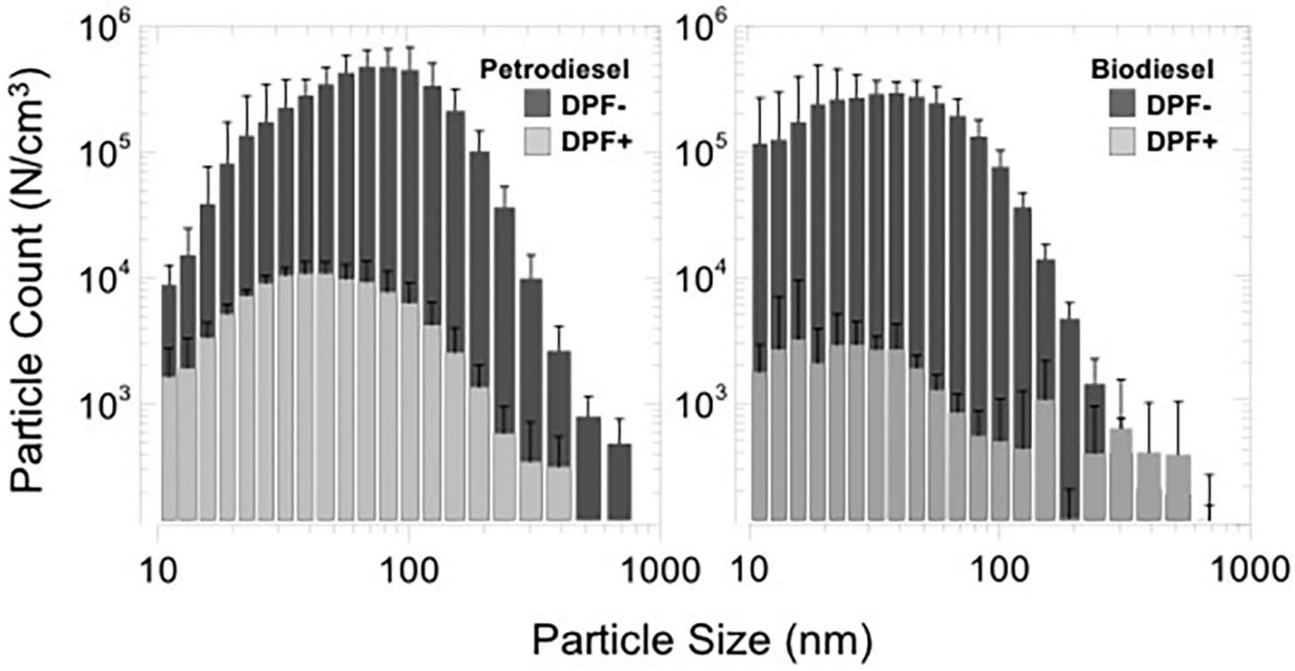

FIG. 1. Particle size distributions for each test condition during cell exposures.

TABLE 1. Count Median Diameter (Geometric Standard Deviation in Parentheses), Count and Mass Concentration in Each Exhaust Treatment

Exhaust Treatment	CMD (nm)	Count Concentration (N/cm^3)	Mass Concentration ($\mu g/m^3$)
Petrodiesel, DPF−	64 (± 2)	6.9E7 (± 1.8E6)	850.3 (± 125.9)
Petrodiesel, DPF+	43 (± 2)	1.4E5 (± 3.2E4)	35.3 (± 6.6)
Biodiesel, DPF−	32 (± 2)	5.9E5 (± 3.7E5)	235.6 (± 8.8)
Biodiesel, DPF+	25 (± 2)	5.8E4 (± 3.4E4)	21.7 (± 1.4)

Note. Data shown are averages; one standard deviation is shown in parentheses.

TABLE 2. Carbon Content of Emissions in Each Exhaust Treatment

Exhaust Treatment	Mass Concentration		Carbon Fractions	
	OC ($\mu g/m^3$)	EC ($\mu g/m^3$)	Percent OC, OC/TC	Percent EC, EC/TC
Petrodiesel, DPF−	186.7 (± 88.2)	595.8 (± 2.5)	24% (± 18%)	76% (± 14%)
Petrodiesel, DPF+	8.8 (± 11.0)	6.2 (± 5.7)	59% (± 33%)	41% (± 40%)
Biodiesel, DPF−	93.5 (± 13.3)	65.9 (± 7.2)	59% (± 8%)	41% (± 24%)
Biodiesel, DPF+	7.7 (± 8.8)	2.1 (± 2.5)	79% (± 43%)	21%(± 18%)

Note. Values are averages, and one standard deviation is shown in parentheses. OC and EC content represent the organic and elemental carbon content, respectively.

TABLE 3. Gaseous Pollutant Concentrations During Cell Exposures

Exhaust Treatment	CO (ppm)	CO_2 (ppm)	NO_x (ppm)	NO (ppm)	NO_2 (ppm)	HC (ppm)
Petrodiesel, DPF−	5.7 (± 0.50)	0.31 (± 0.017)	21.0 (± 0.0428)	19.0 (± 0.411)	2.30 (± 0.368)	3.1 (± 0.048)
Petrodiesel, DPF+	0.13 (± 0.015)	0.30 (± 0.0022)	20.2 (± 0.206)	10.1 (± 0.197)	10.1 (± 0.0088)	0.42 (± 0.029)
Biodiesel, DPF−	3.4 (± 0.036)	0.26 (± 0.05)	13.1 (± 11.3)	11.7 (± 9.60)	1.41 (± 1.67)	2.0 (± 0.0077)
Biodiesel, DPF+	0.08 (± 0.001)	0.29 (± 0.0051)	22.6 (± 0.0939)	13.0 (± 0.628)	9.55 (± 0.534)	0.26 (± 0.055)

Note. Values are averages; one standard deviation is shown in parentheses.

delivered varied by 16- (B99, DPF−) to 80-fold (Petro, DPF−), relative to the lowest exposure group, the filtered biodiesel exhaust.

Cellular Response to DE Exposures

Relative transcript production by NHBE cells exposed to whole DE is shown in Figure 2. An exposure-response relationship is evident, with no significant increases in transcript production at 5 min and substantially higher production at 60 min. No significant differences were observed between the responses of petrodiesel or biodiesel exhaust exposed cells. The response in DPF-treated-exhaust-exposed cells was as great, or greater than the unfiltered exhaust exposed cells; the high (60 min) exposure group showed greater increases in the filtered (DPF+) exposure groups. On average, a 60-min exposure to filtered, petrodiesel, and unfiltered, petrodiesel exhaust produced 14.1 ($p < 0.0001$) and 8.8 ($p < 0.0001$) fold increases in CYP1A1 (Fig. 2C), respectively, and 8.3 ($p < 0.0001$) and 4.5 ($p < 0.0001$) fold increases in HO-1, respectively (Fig. 2A). Filtered petrodiesel exhaust resulted in transcript production levels that were 1.6 (CYP1A1) to 1.8 (HO-1) fold greater than transcript production levels in unfiltered exhaust. Filtered biodiesel exhaust followed a similar pattern. Significant differences in transcript production were also observed after 20 min of exposure. HO-1 was significantly increased by a factor of 2.2 ($p = 0.03$) in NHBE cells exposed to unfiltered biodiesel exhaust (Fig. 2B). CYP1A1 increased by a factor of 2.1 ($p = 0.004$) in unfiltered petrodiesel tests (Fig. 2D). No significant increases in cytotoxicity were observed at 2 h after exposure in any of the treatment groups (Fig. 3).

DISCUSSION

The cellular exposure levels and the exposure system used for this work were more representative of "real-world" conditions than previously reported in vitro. We observed statistically significant cellular responses at levels of PM exposure that were 60 to 100,000 fold less than previous studies that exposed cells resuspended extracts of PM collected onto filters (20–200 μg per cm^2 or 20–200 μg per ml; Baulig *et al.*, 2003; Cao *et al.*, 2007; Schwarze *et al.*, 2013; Totlandsdal *et al.*, 2010). Further, the cellular PM exposure levels reported here are similar to the estimates reported by Gangwal *et al.*, for PM deposition in the lungs of a human exposed acutely to a PM concentration of 0.1 mg per m^3 (Gangwal *et al.*, 2011). Using an MPPD model, Gangwal *et al.* estimated that an acute, 24-h exposure to PM concentration of 0.1 mg per m^3 would result in PM deposition of 0.006 to 0.02 μg per cm^2 of lung cellular surface (Gangwal *et al.*, 2011). Our work also aligns with the recent *in vivo* study by Karthikeyan *et al.*(2013), who found that ultrafine particulate matter and increased nitrogen dioxide in DPF treated exhaust may heighten the injury and inflammation after inhalational exposure.

We found that although the particle emissions were greatly reduced in biodiesel and DPF-treated exhaust, the cellular response was not mitigated relative to such reductions. Our work suggests that current exhaust treatment technologies like a diesel particulate filter, and alternative biobased fuels, may not be sufficient in mitigating the negative health outcomes associated with DE inhalational exposure. Similar to the results reported by Karthikeyan, the results reported here also highlight the importance of including gas-phase and semi-volatile organics when studying the toxicity of DE *in vitro* and *in vivo*.

We observed elevated production of transcripts associated with oxidative stress and tumorigenic potential following NHBE exposure to DPF-treated exhaust even though these levels were 8-fold lower than the unfiltered exhaust. Interestingly, most of the PM mass decrease in DPF-treated exhaust came from a decrease in elemental carbon (99% to 97% for petro- and biodiesel, respectively). Previous work by Bonvallot *et al.* found that the elemental carbon core of DPM produced a very weak effect in HBE cells, with regard to oxidative stress, inflammatory response, and PAH metabolism (Bonvallot *et al.*, 2001). On the other hand, the organic carbon fraction, and "native" (carbon core with organic carbon surface) DPM produced strong effects on NHBE oxidative stress, inflammatory response, and PAH metabolism (Bonvallot *et al.*, 2001). Similar to the observations by Bonvallot *et al.*, we found that DPF-treated exhaust produced substantially lower PM mass emissions. However we found that the reduction in mass emissions, due mostly to a reduction in elemental carbon mass emissions, did not coincide with a similar decrease in cellular production of HO-1 and CYP1A1.

DE is a generalized term for hundreds of gaseous and particulate matter species emanating from a diesel engine. The composition of DE can vary greatly with engine loading, fuel type, and exhaust after-treatment. We observed changes in particle size distribution, particle mass concentration, relative carbon content, and gaseous emissions from a heavy-duty diesel engine operated with varying fuel and exhaust treatment, but at similar speed and load (2400 rpm, 107 bhp). Particle emissions, by mass, count, and size, were greatly reduced in both biodiesel and DPF-treated exhaust, when compared with petrodiesel and unfiltered exhaust, respectively. With the exception of nitrogen dioxide, hazardous gaseous emissions were found to decrease in DPF-treated exhaust as well.

We chose to report cellular exposure levels in terms of deposited DPM mass, because particulate mass is an often used metric by which engines are deemed "clean" or "dirty". This metric also allows comparisons to be drawn between our results and previous studies (Attfield *et al.*, 2012; Silverman *et al.*, 2012). In reality, no single exposure parameter is likely adequate to describe the effective dose and subsequent biological effects of DE (Paur *et al.*, 2011; Teeguarden *et al.*, 2007). Although previous work suggests that PM mass emissions in DE may not be the best metric for predicting human health outcomes, metrics such as particle number or surface area concentration also have issues (Cauda *et al.*, 2012; Karthikeyan *et al.*, 2013; Park *et al.*, 2010; Rudell *et al.*, 1999).

We found that median particle size and number concentration decreased with DPF treatment and with biodiesel fuel. As seen in Table 1, the CMD for DPF-treated exhaust was 43 nm (± 1.92) for petro-diesel and 25 nm (± 1.85) for biodiesel exhaust; the CMDs for unfiltered exhaust were larger: 64 nm (± 1.82) and 32 nm (± 1.68), respectively. Surface reactivity increases exponentially with decreasing particle size (Oberdörster *et al.*, 2005). Surface reactivity is attributed to trace metals and redox-active organic chemicals that cause cellular dysfunction and oxidative stress (Geller *et al.*, 2006) and exposure to DE results in oxidative stress in the human airways (Benbrahim-Tallaa *et al.*, 2012; Li *et al.*, 2002). Disruption of the mitochondrial membrane and redox-cycling chemicals in DE (e.g., PAHs, quinones, ketones, metals, and aldehydes) contribute to the formation of reactive oxygen species (ROS) in the cell. Increased ROS can then create reactive metabolites that lead to the harmful, cytotoxic effects from DE exposure (Li *et al.*, 2003). The Nuclear factor-like 2 (Nrf2) pathway is a primary cellular defense against the cytotoxic effects of oxidative stress (Nguyen *et al.*, 2009). DE exposure, and subsequent ROS formation, activates the Nrf2 pathway, which then activates many cytoprotective genes, including HO-1 (Zhang *et al.*, 2005). Increased ROS also activates NFkβ and JAK-STAT pathways and these pathways also result in an in-

TABLE 4. Estimates of Particle Deposition, by Mass, During Each Cell Exposure Treatment

Exhaust Treatment	Deposited DPM Mass (ng per cm^2)		
	5-min exposure	20-min exposure	60-min exposure
Petrodiesel, DPF−	20–30	80–110	230–340
Petrodiesel, DPF+	0.23–0.34	1.0–1.5	3.0–4.0
Biodiesel, DPF−	3.7–4.0	10–20	40–50
Biodiesel, DPF+	0.12–0.23	0.7–0.8	2.0–4.0

Note. Values shown represent the range of deposited PM mass per cellular growth area (Hawley *et al.*, 2014).

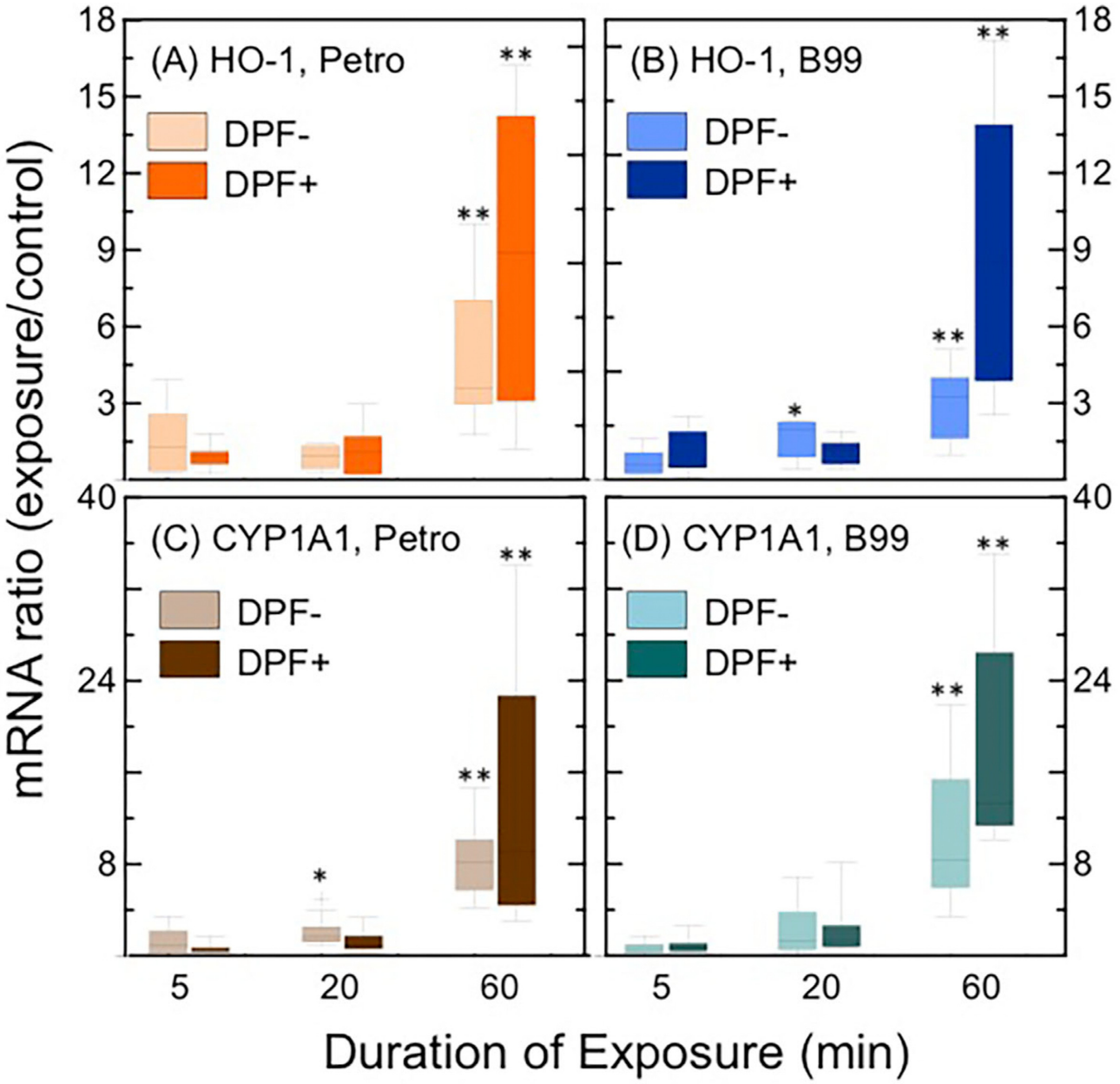

FIG. 2. Box-whisker plots of transcript production in ALI NHBE cells exposed to fresh DE for 5, 20, or 60 min. Panels (A) and (B): HO-1 transcript production in Petro- or B99-exposed cells, respectively. Panels (C) and (D): CYP1A1 transcript production in Petro- or B99-exposed cells, respectively. All transcript levels are normalized to HEPA-air controls. * signifies $p < 0.05$, ** signifies $p < 0.0001$, when compared with controls.

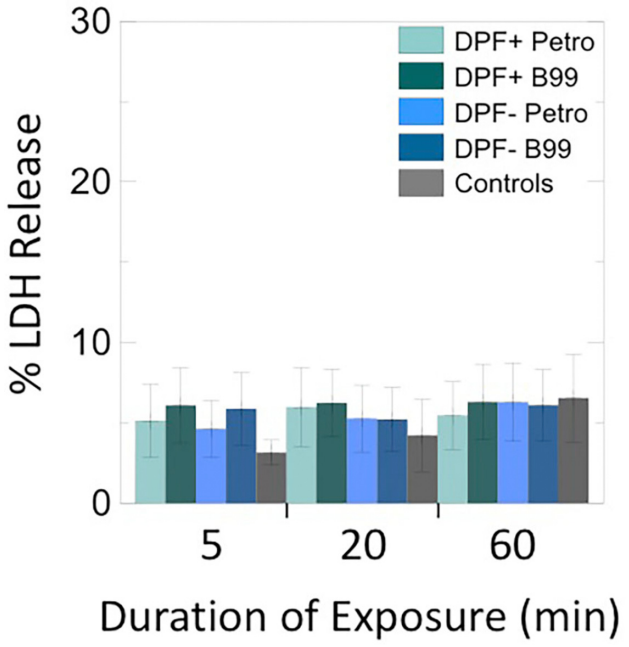

FIG. 3. LDH release from ALI NHBE cells exposed to fresh, complete DE or HEPA-room air with and without an alternating 4-kV electric field. Error bars indicate one standard deviation.

creased production of HO-1 (Liu, 2011; Madamanchi *et al.*, 2001). Further, NO_2 has been observed to be a potent pro-inflammatory mediator and oxidant in previous studies with both bronchial cells and mice (Ayyagari *et al.*, 2007; Johnston *et al.*, 2000). Increased production of transcripts for heme oxygenase-1 has previously been observed in mice exposed to NO_2 at levels three times greater than the high end reported here (30 ppm) (Johnston *et al.*, 2000). Unsurprisingly, we observed an increase in transcript production for HO-1 immediately after exposure to DE (Fig. 2A). We also observed an enhanced response in cells exposed to DPF-treated exhaust. Previous work by Li *et al.* suggests that the polar organic fraction (which contains quinones and oxygenated PAH compounds) was responsible for a subsequent increase in HO-1 in human macrophages (Li *et al.*, 2002). We also note that nitrogen dioxide, a potent oxidant, was increased in DPF-treated exhaust. Although organic carbon mass emissions were decreased substantially in filtered exhaust (95% and 92% for petro- and biodiesel, respectively), we believe that the increased nitrogen dioxide and the remaining ultrafine PM and organic carbon content in filtered exhaust likely contributed to the increased production of HO-1 in NHBE cells exposed to DPF-treated exhaust.

Organic carbon present on the surface of DPM can also carry mutagenic and carcinogenic compounds such as PAHs (Shah *et al.*, 2004). The World Health Organization's International Agency for Research on Cancer recently announced that exposure to DE causes lung cancer in humans (IARC, 2012; Rudell *et al.*, 1999). We chose to measure CYP1A1 because toxic PAH metabolites are formed as a result of cytochrome p450 1A1 (CYP1A1) activity. CYP1A1's function is to increase the solubility of PAHs and thereby increase excretion of PAHs from the body. However, in the conversion of PAHs into more soluble molecules, CYP1A1 creates highly reactive, carcinogenic metabolites (Totlandsdal *et al.*, 2010). CYP1A1 is induced after exposure to PAHs and nitro-PAHs in DE, and the induction of CYP1A1 is thought to contribute to DPM's carcinogenic potential (Jacquet *et al.*, 1996; Ma and Lu, 2007; Mollerup *et al.*, 1999). Further, PAHs and nitro-substituted PAHs found in diesel are thought to increase the risk for lung cancer via the formation of PAH-DNA adducts and mutations in exposed cells and tissues (Chaudhuri, 2013; Lewtas, 2007).

Lu *et al.* observed that gas-phase PAHs make-up the largest fraction of total PAHs in filtered exhaust and that the gas-phase PAHs are often ignored in studies that rely on particle extractions (Hu *et al.*, 2013). Here, exposure to complete, fresh filtered and unfiltered DE led to a large increase in CYP1A1, with greater increases observed in DPF-treated exhaust. Similar to the results observed for transcript production of HO-1, we hypothesize that the increase in CYP1A1 transcript production in NHBE cells exposed to DPF treated exhaust was due to the remaining unbound, gas-phase PAH compounds, alongside of an increase in the potent gaseous oxidant, nitrogen dioxide. Future work designed to measure the gas-phase PAHs and nitro PAHs in unfiltered exhaust and exhaust filtered with a similar DPF, and passive regeneration process, is needed to elucidate the role of the unbound aromatic hydrocarbons in the cellular responses observed here.

Although NHBE cells constitute the majority of the surface of bronchial epithelium, our *in vitro* model is limited. In the bronchial lung, the immediate epithelial response would be followed by the recruitment of neutrophils, macrophages, and fibroblasts. At present, our culture model does not capture the entirety of the response that would be observed *in vivo*. However, we note that our observations here support the observations observed by several *in vivo* studies (Karthikeyan *et al.*, 2013; Rudell *et al.*, 1999). Unlike "traditional" *in vitro* studies, which rely on particle extractions from filters as a source of DPM, our system was capable of exposing cells to complete exhaust (gas-phase PAH and nitro-PAH, quinones, and gaseous emissions) (Gerlofs-Nijland *et al.*, 2013; Pezzulo *et al.*, 2010). Our results differ with those of Gerlofs-Nijland *et al.*, who similarly observed that a biodiesel blend did not reduce the observed cellular response, but alternatively, Gerlofs-Nijland *et al.* observed that DPF-treated DPM contained less cytotoxic, oxidative, and pro-inflammatory potential (Gerlofs-Nijland *et al.*, 2013). However, their study used only extracted DPM, not *complete* exhaust. A growing body of evidence suggests that semi-volatile organic compounds (PAHs, nitro-PAHs, and PAH-quinones) and gaseous emissions (NO_2) are critical to the toxicity observed following exposure to DPF-treated exhaust (Rudell *et al.*, 1999). For example Karthikeyan *et al.* and Rudell *et al.* who exposed rats and humans, respectively, to treated or unfiltered exhaust both reported that a DPF was not sufficient in abolishing the pro-inflammatory effects of the exhaust (Karthikeyan *et al.*, 2013; Rudell *et al.*, 1999). We note that our results should not be generalized to all treatment technologies as different emissions control techniques may produce different particle characteristics from those tested here. Although our *in vitro* model is limited to the human bronchial epithelium, our results from exposing a representative model of the bronchial lung epithelium to complete, fresh DE, support previous *in vivo* studies. Our results suggest that biodiesel and filter-treated DE elicits as great, or greater a cellular response as unfiltered, traditional petrodiesel exhaust.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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